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13. ABSTRACT *(Maximum 200*

Amphiphysin, a neuronal protein first identified in chicken synaptic membranes, is the autoantigen of Stiff-Man syndrome (SMS) associated with breast cancer. During the 3 year course of this fellowship, two isoforms of amphiphysin were cloned and specific mono and polyclonal antibodies prepared. Amphiphysin I was shown to interact specifically with dynamin, a protein known to be important for endocytosis. Amphiphysin II was shown to be concentrated in the cortical cytomatrix of axon initial segments and nodes of Ranvier in brain and around T tubules in skeletal muscle. In the final year of this fellowship, an ELISA assay was developed in order to check for the presence of antibodies to amphiphysin I and II in the sera of breast cancer patients. Though antibodies to amphiphysin I could be readily detected in sera of patients with breast cancer and SMS, no antibodies could be detected from other breast cancer patients.

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PI - Signature 7/15/97 Date

TABLE OF CONTENTS

INTRODUCTION

Stiff-man syndrome (SMS) is a rare neurological disease characterized by rigidity of the body musculature with superimposed painful spasms (Layzer, 1988). Most patients with this disease exhibit autoimmunity to GABA-ergic neurons. It has been found in Prof. De Camilli's laboratory that, to date, all patients diagnosed with breast cancer and SMS, have autoantibodies against a novel synaptic-associated protein, amphiphysin. This research project is to further define the role of anti-amphiphysin antibodies in the development and/or detection of breast cancer and to further understand the function of this protein and how the autoimmunity may arise.

In the early stages of the work on SMS, it was noticed in Prof. De Camilli's lab that two patients with this condition, but without GAD-antibodies or associated organ-specific autoimmune diseases, had high titers of autoantibodies directed against a 128 kDa protein. Immunocytochemistry suggested a synaptic localization of the autoantigen. Interestingly, both patients were women with breast cancer (ductal adenocarcinoma). Subsequently, the same antibodies were detected in a third patient with SMS without apparent breast cancer. On Prof. De Camilli's indication, a search of breast cancer in this patient was performed by ultrasonography and a small infiltrating ductal carcinoma was found and removed. A summary of the caseload of amphiphysin autoimmunity is indicated in table 1. In at least three of these cases (Folli et al, 1993; Meinck, personal communication) a remission of the neurological symptoms was documented after removal of the cancer and steroid therapy, supporting the hypothesis that no major degeneration of brain tissue occurs in SMS.

Table 1: Paraneoplastic Stiff-man Syndrome Cases

	Anti-GAD Autoantibodies	Anti-amphiphysin Autoantibodies	Cancer	Source
Patient 1 England	Negative	Positive	Breast Cancer	Our caseload (Folli et al. 1993)
Patient 2 Italy	Negative	Positive	Breast Cancer	Our caseload (Folli et al. 1993)
Patient 3 Italy	Negative	Positive	Breast Cancer	Our caseload (Folli et al. 1993)
Patient 4 Germany	Negative	Positive	Breast Cancer	Our caseload (De Camilli et al. 1993)
Patient 5 U.SA.	Negative	Positive	Breast Cancer	Our caseload (David et al. 1994)
Patient 6 Germany	Negative	Positive	Breast nodule	Our caseload
Patient 7 U.SA	Negative	Positive*	Breast Cancer	D. Kaufman, personal communication
Patient 8 Italy	Negative	Positive*	Colon Cancer	Grimaldi et al. 1993
Patient 9 Japan	Negative	Positive	Breast Cancer	Tsutsui et al, 1995

*not tested in our lab

These findings raise the possibility that in some cases SMS may have an autoimmune paraneoplastic origin. Other autoimmune paraneoplastic neurological diseases have been described and characterized in recent years (Posner and Furneaux, 1990; Hetzel et al, 1990). These conditions are characterized by neurological symptoms which appear to follow the development of a cancer, and by the presence in the serum and CSF of high titer antibodies directed against specific brain autoantigens. The type of antibodies generally correlate with the type of neurological symptoms, but the pathogenic role of these antibodies remains unclear. It was proposed that ectopic expression of brain antigens by cancer cells triggers the immune response (Furneaux et al, 1990).

Amphiphysin is a synaptic-vesicle-associated protein that was discovered by the screening of a λGT11 library of chicken brain with antibodies to synaptic proteins (Lichte et al, 1992). Its sequence (total of 682 amino-acids) includes a stretch of about 20 amino-acids which could potentially form a transmembrane span. However, most of the protein is cytosolic and only a pool of the protein interacts with the cytoplasmic surface of synaptic vesicles. Its function is unknown. The properties of amphiphysin suggested a possible identity with the 128 kDa antigen, a hypothesis that was tested and confirmed (De Camilli et al, 1993).

We have been able to clone human amphiphysin and found the N- and C-terminal domains of the protein to be highly conserved between chicken and human (David et al, 1994). Patient autoantibodies have a distinct pattern of reactivity with amphiphysin, and the dominant autoepitope is located in its C-terminal region, which contains an SH3 domain (David et al, 1994). Portions of chicken and human amphiphysin are also homologous to portions of Rvs167 and Rvs161 (David et al, 1994), two yeast proteins whose mutant phenotype includes a striking

endocytic defect (Munn et al, 1995) in addition to growth and polarity defects (Crouzet et al, 1991; Bauer et al, 1993; Desfarges et al, 1993).

We have demonstrated a specific, SH3 domain-mediated, interaction between amphiphysin and dynamin by gel overlay and affinity chromatography (David et al, 1996). In addition, we showed that the two proteins are colocalized in nerve terminals and are coprecipitated from brain extracts consistent with their interactions in situ. We also reported that a region of amphiphysin distinct from its SH3 domain mediates its binding to the α_C subunit of AP2 adaptin, which is also concentrated in nerve terminals (David et al, 1996). These findings support a role of amphiphysin in synaptic vesicle endocytosis.

The current work was aimed at understanding more about amphiphysin function in the brain and to test the possibility that amphiphysin or a related protein may play a role in the biology of breast cancer.

BODY

Task 1, Preparation of recombinant amphiphysin and specific antibodies to it

a. Recombinant amphiphysin will be injected into rabbits and mice for production of polyclonal and monoclonal antibodies

This aspect of the project was addressed during years one and two of the fellowship.

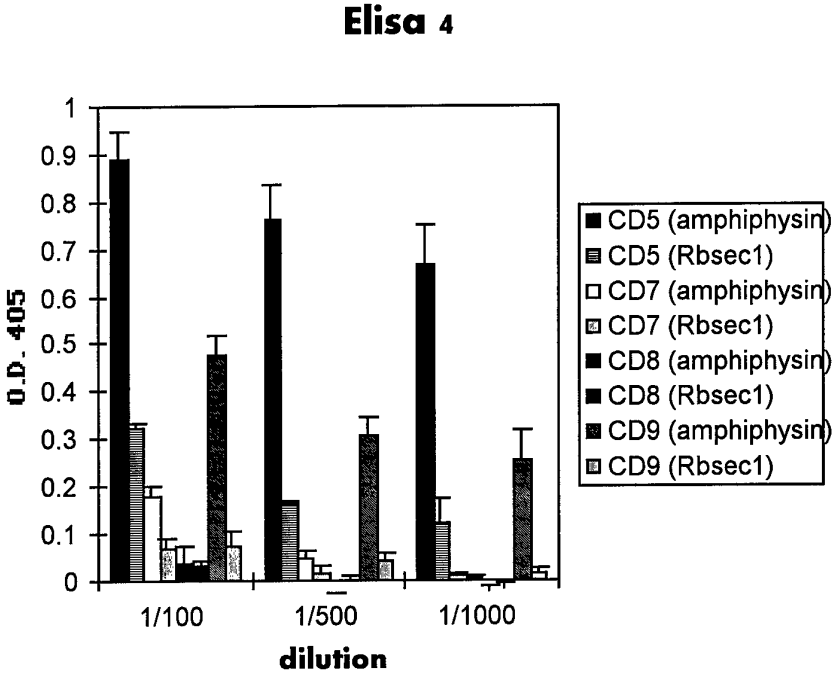
Task 2, Developing a screening assay to check for amphiphysin autoimmunity in a large population of breast cancer patients

Method: An ELISA assay was developed using recombinant His-tagged amphiphysin as an antigen in order to check for anti-amphiphysin antibodies in the sera of breast cancer patients. Wells (Nunc immunoplate Maxisorp) were coated with recombinant His-amphiphysin I (David et al, 1996) at 10μg/ml in PBS (o.n. 37^0C). In parallel, serum dilutions were prepared in a blocking solution of 3% BSA/20% DH5α lysate in PBS and were incubated on a roller at 4^0C o.n. The next day, plates were blocked with 250μl 3% BSA in PBS (1 hour, r.t.). They were then washed twice with PBS and then incubated for 2 hours (r.t) with 50 ml of the antibody/bacterial lysate solution. Wells were washed 3 x with PBS and incubated with alkaline phosphatase-coupled secondary antibody and developed with p-nitrophenyl phosphate tablets (Sigma). The O.D. of the wells was read at 405nm with an ELISA plate reader. For non-specific reactivity a His-tagged fusion protein of rbsec1A (Garcia et al, 1994) was used.

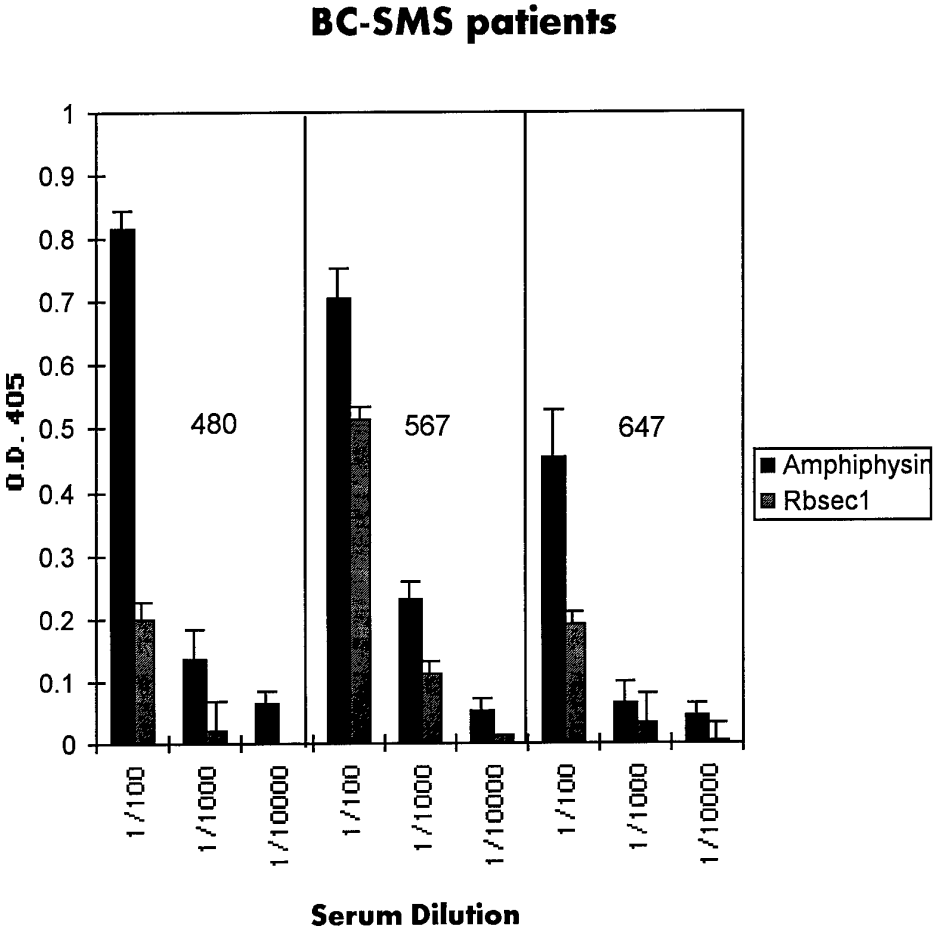
A similar assay was developed using amphiphysin II as an autoantigen. To this end, a GST fusion protein of the amphiphysin II (clone 17/19, Butler et al, submitted) was made and purified on GTH beads. GST alone was used to check non-specific reactivity.

Results:

Anti-amphiphysin I antibodies can be detected in control sera from rabbits injected with amphiphysin or from patients with breast cancer and stiff-man syndrome.

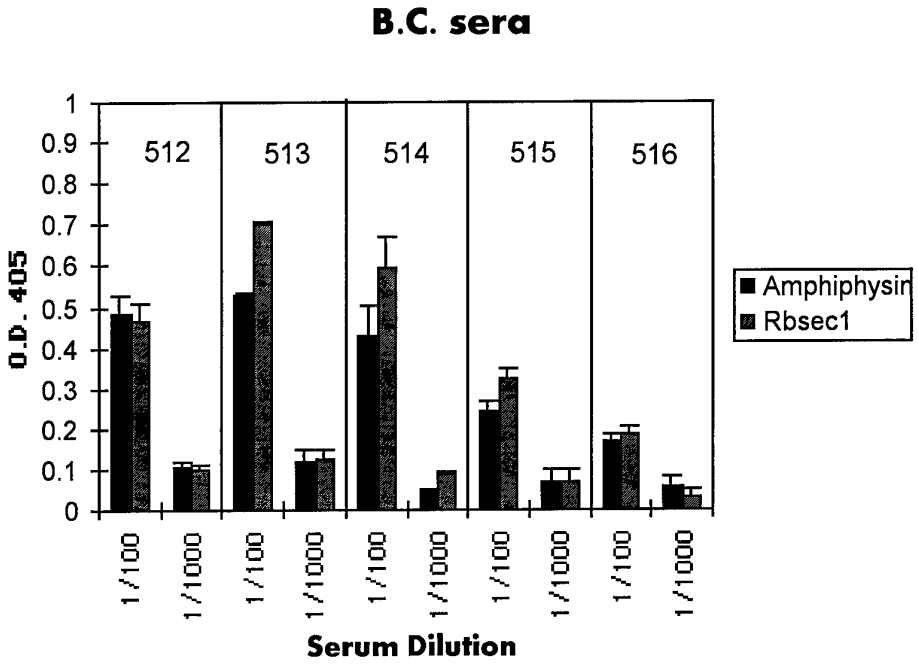


Anti-amphiphysin I antibodies were detected in sera from rabbits CD5 (injected with recombinant amphiphysin I) and CD9 (injected with a synthetic peptide from amphiphysin I and II) but not in sera from rabbits CD7 and CD8 (injected with recombinant amphiphysin II).



Anti-amphiphysin I antibodies were detected in sera from patients with breast cancer and stiff-man syndrome.

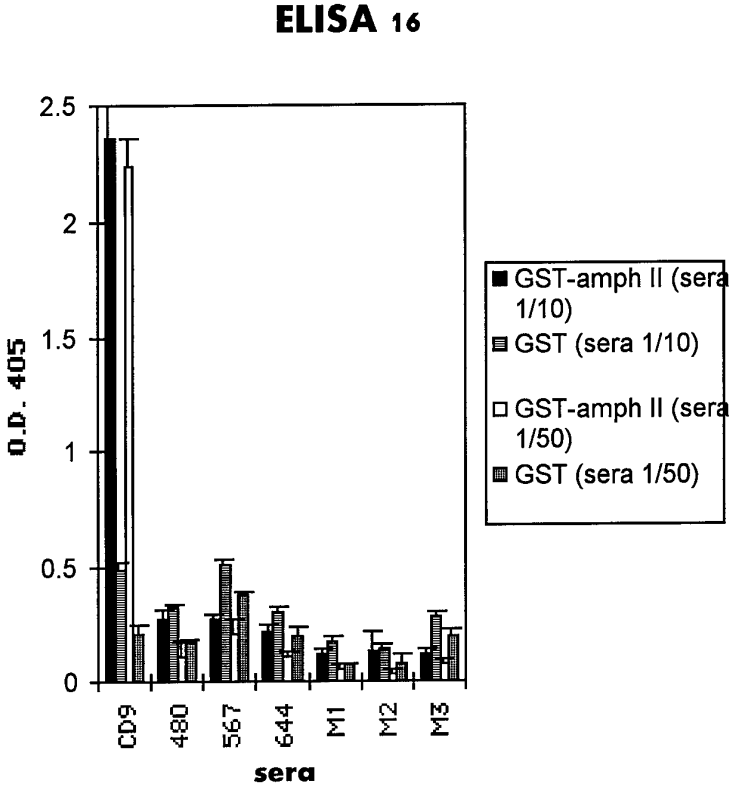
No anti-amphiphysin antibodies were detected in sera from breast cancer patients without stiff-man syndrome.



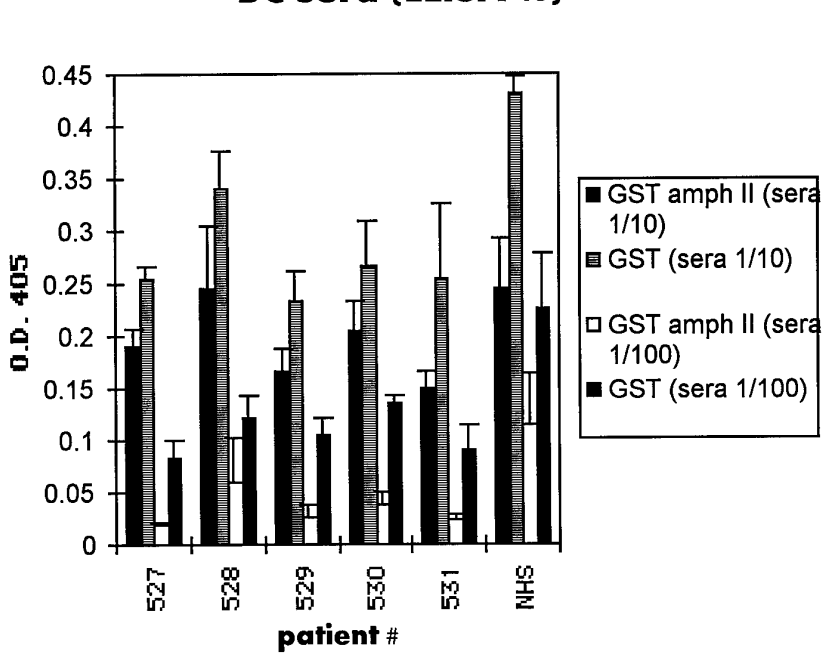
A representative ELISA assay of breast cancer sera. The results are shown for 5 of 20 breast cancer sera that were checked for the presence of anti-amphiphysin I antibodies. The sera show reactivity to both amphiphysin and the control protein, Rbsec1.

Mouse sera was taken from mice that had been transplanted with human breast cancer cells and had growing tumors (kind gift of H. Degani, Weizmann Institute of Science). No anti-amphiphysin I antibodies were detected in their sera.

Detection of amphiphysin II antibodies: Amphiphysin II antibodies could be detected in control CD9 sera (rabbit) but not in any of the patient sera, from breast cancer patients with or without stiff-man syndrome.



Anti-amphiphysin II antibodies were detected in sera from rabbit CD9 but not in the sera form BC-SMS patients or from mice with transplanted human tumors.



Task 3, Studying the function of amphiphysin and how it relates to cancer

a. Human amphiphysin will be overexpressed in a variety of cell lines where its phenotype can be studied.

This aspect of the project was addressed in the second year.

b. The proteins that associate with amphiphysin will be identified

This aspect of the project was addressed in year 1 (refer to David et al, 1996).

Studies were also carried out in the third year of the project to determine if proteins that interact with amphiphysin II could be identified. Amphiphysin II has been cloned by a separate group and termed Bin1 (Sakamuro et al, 1996). Bin1 was shown to be a nuclear protein that was postulated to interact with the oncogene, myc. In order to search for proteins that may interact in the nucleus with the SH3 domain of amphiphysin II, nuclear extracts were prepared from rat liver. SDS-PAGE analysis of the extracts was carried out and then overlaid with GST fusion proteins of the SH3 domain of amphiphysin I or II, or GST alone (as in David et al , 1996). A faint band of 15 kDa could be detected with both amphiphysin I and II SH3 domains. This band was not further characterized.

c. The expression of amphiphysin in normal and neoplastic tissue will be studied

This aspect of the project was addressed in year 2.

CONCLUSIONS

Amphiphysin has been identified as an autoantigen in breast cancer when it is accompanied with a rare autoimmune disease called Stiff-Man syndrome. Previously it had been shown that patients with Stiff-Man syndrome and diabetes had antibodies to glutamic acid decarboxylase (GAD). Through these studies, GAD was found to be a major autoantigen in diabetic patients that did not have Stiff-Man syndrome. By analogy, it was quite plausible and of great interest to study whether a larger population of patients with breast cancer may have antibodies to amphiphysin as well. Cloning of amphiphysin I and II isoforms and the expression of large amount s of the purified proteins made this study feasible. In the final year of the fellowship, an ELISA assay was developed in which anti-amphiphysin antibodies could be detected to both isoforms of the protein. Sera were pre-incubated with bacterial lysate in order to decrease the background, but still, no specific reactivity to either amphiphysin I or II could be detected in the breast cancer patients.

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